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Combined effect of copper sulfate and water temperature on key freshwater trophic levels – Approaching potential climatic change scenarios

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ABSTRACT

This work relied on the use microcosms to evaluate the individual and the combined effects of different levels of copper sulfate (0.0, 0.013, 0.064 and 0.318 mg Cu L^{-1}) – a fungicide commonly exceeding allowable thresholds in agricultural areas – and a range of water temperature increase scenarios (15, 20 and 25 °C) on freshwater species belonging to different functional groups. Hence, the growth inhibition of primary producers (the microalgae *Raphidocelis subcapitata* and the macrophyte *Lemna minor*), as well as the survival and feeding behavior of a shredder species (the Trichoptera *Schizopelex* sp.) were evaluated. The results revealed that copper was toxic to primary producers growth, as well as shredders growth and survival, being the growth of *L. minor* particularly affected. Higher water temperatures had generally enhanced the growth of primary producers under non-contaminated (microalgae and macrophytes) or low-contaminated (macrophytes) conditions. Despite the tendency for a more pronounced toxicity of copper under increasing water temperatures, a significant interaction between the two factors was only observed for microalgae. Since the test organisms represent relevant functional groups for sustaining freshwater systems functions, the present results may raise some concerns on the impacts caused by possible future climate change scenarios in aquatic habitats chronically exposed to the frequent or intensive use of the fungicide copper sulfate.

1. Introduction

Copper-based compounds are one of the chemicals most used in the European Union, namely in organic and conventional viticulture to control fungal diseases like downy and powdery mildew. In Europe, viticulture is responsible for copper consumptions between 1883 and 6842 t per year (EUROSTAT, 2007). The large amounts and wide variety of copper-based agrochemicals applied is likely to lead to the contamination of the neighboring water resources, hence affecting their quality in terms of chemical and ecological *status*, which represents a particular challenge for the successful implementation of the Water Framework Directive (EC, 2000).

Copper toxicity was already proved in several studies targeting primary producers (Oliveira-Filho et al., 2004; Khellaf and Zerdaoui, 2009; Zhao et al., 2015), cladocerans (Mastin and Rodgers, 2000; Oliveira-Filho et al., 2004), mussel larvae (Clearwater et al., 2014), shredders (Gama et al., 2014; Zubrod et al., 2014) and fish species (Oliveira-Filho et al., 2004; Haverroth et al., 2015). However, such studies usually fail to integrate other relevant stress factors and/or evaluate their combined impacts on biological responses.

For instance, climate change has been discussed as an additional threatening stress to freshwater resources normally impacted by agricultural diffuse pollution. According to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC, 2013), the annual temperature of Southern European/Mediterranean area is predicted to increase 1.7 (min – max: 0.7 – 3.1 °C, scenario RCP4.5) and 2.3 °C (0.6 – 4.0 °C, RCP4.5) up to the middle and end of the 21st century, respectively. In summer, the predictions indicate even bigger temperature increases, 2.2 (1.0 – 4.3 °C, RCP4.5) and 2.8 °C (1.2 – 5.5 °C, RCP4.5), respectively. These increments may lead to similar increases in the temperatures of surface water bodies. Overall, warming conditions are expected to result in shifts in the hydrologic cycle, depletion of dissolved oxygen levels and increase of the concentrations of nutrients and contaminants (Ficke et al., 2007; Whitehead et al., 2009; Kim et al., 2010; Mas-Martí et al., 2015). In biological terms, higher temperatures tend to accelerate the metabolism of organisms, affect

Abbreviations: ASGR, average specific growth rate; EC, effect concentration; GE, growth efficiency; LC, lethal concentration; LOEC, lowest observed effect concentration; RCR, relative consumption rate; RGR, relative growth rate; ROS, reactive oxygen species

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their respiration rates, growth and development time, together with an increased consumption, decomposition and excretion rates (e.g., Friberg et al., 2009; Dallas and Ross-Gillespie, 2015). Furthermore, a temperature rise affects the sensitivity of organisms to contaminants since, on one hand, the uptake of chemicals by organisms is enhanced, resulting in higher accumulation of toxic compounds. On the other hand, higher rates of chemical reactions may lead to the production of free radicals and biotransformation products that can be more toxic than the parent compounds (Murdoch et al., 2000; Ficke et al., 2007). At a higher ecological level, increased water temperatures are expected to change the geographic distribution of species, hence influencing the overall aquatic biodiversity (Heino et al., 2009; Zhao and Feng, 2015).

In the actual climate change context, it is of crucial importance to anticipate the impact of temperature per se and its effects in combination with other stressors, like copper contamination, in order to improve environmental management and protection strategies in agricultural areas. As such, we hypothesized that the combined action of copper toxicity and temperature variations within the previewed climate change scenarios may affect biological responses of organisms belonging to different trophic levels. To address this hypothesis, the present study relied on the use of microcosm experiments to assess: (i) the toxicity of a copper-based fungicide on three non-target freshwater species – two primary producers (the green algae *Raphidocelis subcapitata* and the macrophyte *Lemna minor*), and one shredder species (the trichoptera *Schizopelex* sp.); (ii) the impact of increased water temperatures linked to climate change scenarios on the biological responses of these organisms; and (iii) the combined effect of these two stress factors on those key species from freshwater ecosystems.

2. Materials and methods

2.1. Stress factors – temperature regimes & copper concentration and analysis

Three constant temperature regimes were established: 15, 20 and 25 °C. The lowest temperature was the temperature of the stream water during the field sampling (cf. Section 2.2.3), corresponding to the baseline scenario. The two higher temperatures are the result from two successive temperature increases of 5 °C, in line with the maximum temperature increases expected for the Southern European/Mediterranean region. The range of temperatures tested covers the current temperatures of Portuguese freshwater systems [8 – 12 °C in Winter (Pascoal et al., 2005; Ferreira et al., 2010), 16 – 21 °C in Spring (Pascoal and Cássio, 2004)] and of the large rivers and lakes in Europe [9 – 21 °C (EEA, 2012)], as well as their future temperatures according to the regional climate change projections.

Copper (as $CuSO_4 \cdot 5H_2O$; Merck) toxicity was assessed at three nominal concentrations: 0.0, 0.013, 0.064 and 0.318 mg Cu L^{-1}, being the stock solution prepared in distilled water. The highest concentration was selected according to the highest concentration of copper detected in Portuguese surface water bodies (0.318 mg Cu L^{-1}, SNIR, 2015), while the other two concentrations corresponded to two successive dilutions by applying a factor of 5. The obtained range of concentrations is representative of the copper levels reported for surface waters from different countries in the vicinity of agricultural lands (0.01 – 0.06 mg L^{-1}, Arbneshi et al., 2008; 0.01 – 0.117 mg L^{-1}, Fernández-Calviño et al., 2008). The copper sulfate stock solutions were maintained at 4 °C for no longer than 72 h until proceeding with the spiking of leaves and water.

The effective copper concentrations were determined immediately before the test by UV–Vis spectrophotometry (adapted from APHA (1992) and Ramadan et al. (2009)). This involved adding trisodium citrate buffer and the complexing agent 4-(2-pyridylazo)-resorcinol (PAR) to three aliquots of each stock solution, and then the copper levels were measured at 539 nm. The nominal copper concentrations of 0.013, 0.064 and 0.318 mg L^{-1} corresponded, on average, to effective concentrations of 0.021, 0.054 and 0.322 mg L^{-1}, respectively. No copper was detected in the stream water used in the experiments.

2.2. Culture of organisms, sampling and processing of biological material

2.2.1. Microalgae culture and immobilization

Unialgal cultures of *R. subcapitata* were maintained in the laboratory in sterile Woods Hole MBL (Marine Biological Laboratory) medium (Stein, 1973). The microalgae were harvested at the exponential growth phase (5 – 7 days old) and inoculated into fresh medium to initiate new microalgae cultures. Microalgae were normally reared at 20 ± 2 °C and a 16 ^{L}: 8^{D} photoperiod. A batch of microalgae cultures were acclimated to continuous light and to the other test temperatures (i.e., 15 and 25 °C), following the acclimatization procedure described for the trichopterans in sub-section 2.2.3.

Microalgae cells were immobilized in a calcium alginate matrix following the procedures outlined in Moreira dos Santos et al. (2002) and Marques et al. (2011). Briefly, for each testing temperature, an aliquot of the temperature-acclimated culture (at the exponential growth phase) was centrifuged and resuspended in MBL. A certain volume of this suspension was added to a solution of sodium alginate 1.3% (w/v) as to obtain an initial cell density of 10^6 cells mL^{-1}. After approximately 30 min of agitation, drops of such suspension of alginate and algal cells were incorporated into a solution of calcium chloride 2% (w/v). After 60 min of agitation to allow the formation and hardening of alginate beads, they were washed in distilled water and kept in 20x diluted MBL medium at 4 °C in the dark, for no longer than 48 h until use in the test.

2.2.2. Macrophytes culture

Colonies of *Lemna minor* were maintained in Erlenmeyers with Steinberg medium (OECD, 2006a), under 20 ± 2 °C and continuous light. The cultures were renewed weekly. Some of them were however acclimated to 15 °C and 25 °C before conducting the microcosm experiments, as previously mentioned (cf. Section 2.2.1).

2.2.3. Sampling and acclimatization of trichopterans

In late winter and beginning of spring season, *Schizopelex* sp. larvae of similar size (ubiquitous trichoptera species in low order Portuguese streams) were collected from the spring of a second order stream (Rio de Castelões, in the Mondego basin) in the Caramulo Mountain, North-Central Portugal (N40°32'0.73" and W8°09'15.79"; 222 m above sea level).

The trichoptera larvae were randomly divided into three aquariums containing aerated stream water and streambed sediment (also collected during the field campaign). The water of the three aquariums was initially maintained at 15 °C (the temperature of the stream water), but two of them were gradually warmed up to 20 and 25 °C at rates of 0.3 and 0.7 °C *per* day, respectively, over a period of 15 days. During this 15-day acclimatization period, the larvae were fed with leaves from a mixture of plant species (cf. Section 2.2.4). The water and sediments in the aquariums were weekly renewed after pre-acclimatization.

2.2.4. Collection, conditioning and contamination of leaf disks

Undamaged chestnut leaves (*Castanea sativa*) were collected at abscission time (in winter) at the banks of the same stream. The chestnut leaves were air dried and stored in a dry and dark place. Later on, leaf disks with 12-mm diameter were obtained from these leaves with a cork borer and then introduced into 0.5 mm mesh bags (20 × 20 cm). These bags were taken to the same stream and immersed in a stream section (in late winter/beginning of spring) with a slow current to promote microbial colonization, thereby enhancing palatability of leaves to detritivores. The bags were recovered after one week of in situ microbial conditioning.

Upon arrival at the laboratory, the leaf disks were gently rinsed with distilled water to remove detritus, and randomly divided into four

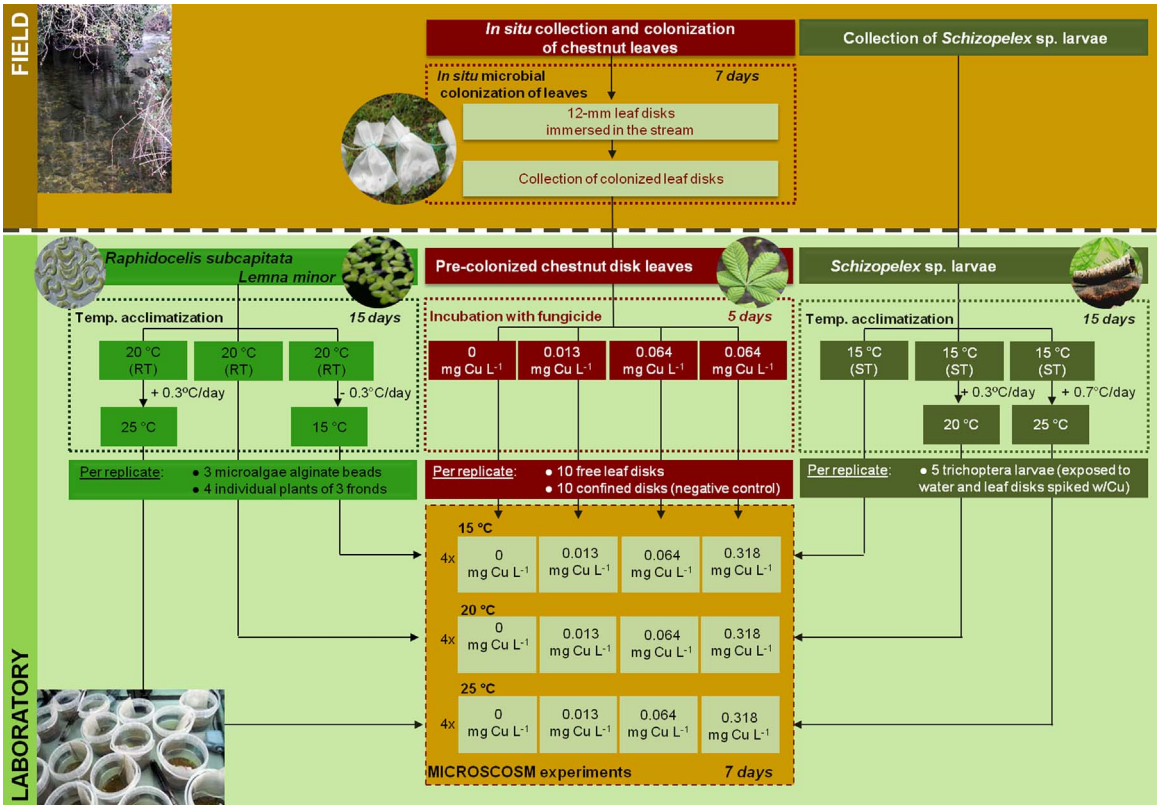

Fig. 1. Schematic diagram describing the experimental approach followed and the design of microcosm trials for testing the combined effect of copper sulfate and temperature on non-target freshwater organisms (*Raphidocelis subcapitata*, *Lemna minor* and *Schizopelex* sp.). *RT* – Room temperature, *ST* – Stream temperature.

groups, after which they were spiked with different copper concentrations. For their contamination, each group of leaf disks was placed in 2 L-Erlenmeyers containing leaves from mixed plant species collected in the streambed as to promote further conditioning, and 1.5 L of stream water spiked with the different copper concentration [see Section 2.1.: 0 (non-contaminated stream water - control), 0.013, 0.064 and 0.318 mg Cu L^{-1}]. The erlenmeyers were incubated for five days at 20 ± 2 °C under continuous mechanical agitation and aeration. Afterwards, the leaf disks were removed and gently washed with distilled water to remove the excess of debris before being used in the microcosm experiments on shredder feeding inhibition (Section 2.2.3).

2.3. Microcosms and toxicity tests

The overall design of the microcosm experimental trials and their preparation steps are schematically represented in Fig. 1. Briefly, the preparation steps involved field work related with the collection of stream water, sediment, trichoptera and chestnut leaves, and the in situ conditioning of the latter. The work in the laboratory involved the maintenance/preparation of the test species, as well as the copper-spiking of water samples (cf. Section 2.1) and leaf disks (cf. Section 2.2.4), and performance of the microcosm trials.

The microcosm assays were conducted in cylindrical plastic vessels (10 cm diameter × 10 cm high) filled with approximately 100 g of sterilized sediment (autoclaved at 120 °C for 45 min) and 0.5 L of stream water. Water spiking with the three copper concentrations was performed before the introduction of the organisms into the microcosm recipients. To each vessel was added: (i) a net bag (0.5 mm pore mesh size) containing three beads of alginate-immobilized microalgae, (ii) four *L. minor* colonies with three fronds (Section 2.3.2), (iii) five *Schizopelex* sp. larvae, (iv) ten pre-conditioned and pre-contaminated leaf disks (free disks) and a 0.5 mm mesh net bag containing ten more pre-conditioned and pre-contaminated leaf disks (confined disks; added to the experiment as a negative control for the feeding inhibition assay, cf. Section 2.3.3). Four microcosm vessels (i.e., replicates) were used for each copper concentration (0.0, 0.013, 0.064 and 0.318 mg Cu L^{-1}) at each temperature regime (15, 20 and 25 °C). In order to test the different temperatures simultaneously, the 16 microcosms of each temperature regime were placed in a separate chamber with its own set of refrigerators, thermostats and pumps for water circulation and water temperature control. All vessels were kept under continuous and average light intensity of 8000 lx.

2.3.1. Microalgae growth inhibition tests

The *R. subcapitata* growth inhibition test followed the basic procedures outlined in the OECD 201 (2006b) with some amendments, namely immobilized cells were used instead of free cells (Moreira dos Santos, 2002; Marques et al., 2011). Twenty beads were considered to determine the initial cell density. At the beginning of the microcosm experiment, three microalgae-immobilized alginate beads were introduced in each microcosm recipient of the respective testing temperature. The immobilized microalgae were exposed to the treatments during 72 h. In order to assess the density of algae cells at the beginning and at the end of the test, the beads were disaggregated with trisodium citrate 3% (w/v) and the cells were microscopically counted using a Neubauer haemocytometer.

2.3.2. Macrophyte growth inhibition tests

The *L. minor* growth inhibition test was performed according to the OECD 221 (2006a) standard with adaptations concerning the exposure system (i.e., microcosms). The test was run for 7 days. As referred above, four individual plants with three fronds from each acclimatized culture were randomly introduced in each microcosm recipient of the respective testing temperature. Four plants with three fronds were used to determine the initial dry weight (105 °C; 48 h), measured at the nearest 0.0001 g (these macrophyte colonies were not used in the

exposures). At the end of the test, the dry weight (105 °C; 48 h) of macrophytes was quantified. The average specific growth rate (ASGR, day^{-1}) of microalgae and macrophytes were determined according the following formula:

$$ASGR_{microalgae} = (Ln\ CD_f - Ln\ CD_i)/\Delta t$$

$$ASGR_{macrophyte} = (Ln\ MM_f - Ln\ MM_i)/\Delta t$$

where CD_f and CD_i are the final and initial algae cell density (cells mL^{-1}), and MM_f and MM_i are the final and initial macrophytes dry mass (g), respectively, after the test period Δt (days).

2.3.3. Shredder feeding inhibition test

At the initiation of the microcosm experiment, five random *Schizopelex* sp. larvae from each acclimatized aquarium were introduced in each microcosm recipient of the respective testing temperature. The average initial dry weights of twenty larvae and of twenty leaf disks were determined after drying them at 105 °C during 48 h. The feeding test run for 7 days, and during this period the mortality of the larvae was daily checked. At the end of the test, besides the survival of larvae (%), the relative growth rate (RGR; $g_{larvae\ DM}\ g_{larvae\ DM}^{-1}\ day^{-1}$), relative consumption rates (RCR; $g_{disk\ DM}\ g_{larvae\ DM}^{-1}\ day^{-1}$), and growth efficiency (GE; %) were determined according the following formulas:

$$RGR = (L_f - L_i)/(L_f * t)$$

$$RCR = D_e/(L_f * \text{test duration})$$

$$GE(\%) = (L_f - L_i/D_e) * 100,$$

where L_f and L_i refer to the final and initial larvae dry mass (g), D_e to the dry mass of the disks eaten by the invertebrates (g) and t to the exposure time (days). D_e was calculated as:

$$D_e = (D_i - D_f)/(D_i * ((Cd_i - Cd_f)/Cd_i)),$$

where D_i and D_f refer to the initial and final dry masses of free disks, and Cd_i and Cd_f to the initial and final dry masses of confined disks.

2.4. Data analysis

Two-way analyses of variance (ANOVAs) were applied to test for the significance of the factors and their interaction on the responses of the test organisms. The arcsine square root transformation was applied to the mortality data of *Schizopelex* sp. to avoid violating the underlying assumption of normality. In the absence of a significant interaction between the factors, the effect of each one was analyzed by the Tukey test. In the presence of significant interactions, the influence of the individual factors on biological responses was explored by simple main effects analysis. In the main effects analysis, the $MS_{residual}$ of the two-way ANOVA was used as denominator for calculating the F value of the one-way ANOVA and the q statistics of the Tukey test applied to each factor (Quinn and Keough, 2002). In all the analyses, the significance level (α) considered was 0.05.

The EC_x values and corresponding 95% confidence limits were determined by non-linear estimation, using a logistic model fitted to the data through the least squares method. For larvae mortality, the LC_x values and corresponding 95% confidence limits were estimated by Probit analysis (Finney, 1971).

3. Results

3.1. Primary producers

Copper sulfate contamination and the interaction with increased water temperatures induced effects on the growth of *R. subcapitata* (Table 1). Significant reductions on the microalgae growth rates, comparatively to the respective control, were observed at 0.064 and 0.318 mg Cu L^{-1} both under 20 and 25 °C (Fig. 2). This temperature-dependent increasing toxicity was apparently corroborated by the EC_{10} and EC_{20} values. Both values differed more than or nearly to one order of magnitude from 20 to 25 °C (Table 2A). The increase of water temperature resulted in higher growth rates in the control at 20 °C and especially at 25 °C (Fig. 2).

Table 1
Summary of the two-way ANOVA applied to the average specific growth rate (ASGR) data of the microalgae *Raphidocelis subcapitata* and the macrophyte *Lemna minor*. Significant effects are signed out in bold ($p < 0.05$).

Species	Endpoint	Source of variation	df	MS	F	p
R. subcapitata	ASGR	Copper contamination	3	0.113	45.738	< **0.001**
		Temperature	2	0.002	0.773	0.469
		Copper x Temperature	6	0.01	4.053	**0.003**
		Residual	36	0.002	–	–
L. *minor*	ASGR	Copper contamination	3	0.042	19.745	< **0.001**
		Temperature	2	0.016	7.691	**0.002**
		Copper x Temperature	6	0.001	0.314	0.925
		Residual	36	0.002	–	–

The *L. minor* growth results partly corroborated the microalgae response (Fig. 2). Higher concentrations of copper sulfate were responsible for statistically significant reductions of macrophytes growth (not only at 20 and 25 °C, as observed with microalgae, but also at the lowest temperature of 15 °C), though for this species the interaction between the two stress factors was not significant (Table 1). In the control and in the lowest contamination scenario (0.013 mg Cu L^{-1}) the warmer conditions resulted in significant higher growth rates of the macrophytes (Table 1, Fig. 2). Contrary to the response of microalgae, the EC_x values calculated for the macrophytes growth were generally higher at warmer conditions (20 and 25 °C), especially at 20 °C (Table 2A).

3.2. Shredder

No mortality of *Schizopelex* sp. larvae was observed in the control treatments, as well as in the lowest copper sulfate concentration (0.013 mg Cu L^{-1}). Notwithstanding, there was a statistically significant reduction of larvae survival at the highest levels of copper, particularly at warmer temperatures, as suggested by the generally reduced LC_x values at 20 °C and 25 °C (Fig. 3, Tables 3 and 4A). Hence, both stress factors were apparently responsible for the observed acute effects, despite the absence of a significant interaction between them (Table 3).

The RCRs, the RGR and the GE were significantly affected by copper contamination, but not by water temperature, nor a significant interaction was observed (Fig. 3, Table 3). Larvae RCRs were stimulated under copper spiked-disk leaves and water, attaining the most elevated and significant values under 0.318 mg Cu L^{-1}, both at 20 and 25 °C. Since there was a stimulation of RCR, the EC_x values responsible for deleterious effects in this parameter were not determined.

In turn, the contamination of food and water with copper sulfate produced a significant depletion on RGR of larvae was observed, irrespectively of the temperature regime (Fig. 3, Table 3). These results were strengthened by the similar EC_x values between temperatures, though a slightly higher toxicity of copper was noticed at 20 °C based on the EC_{20} results (Table 4A).

The GE had a similar pattern to that of RGR, being also achieved similar results in the two-way ANOVA (Table 3). The GE of larvae was reduced under increasing copper sulfate concentrations in all temperatures, though a significant decrease was only observed at 20 and 25 °C (Fig. 3). Again at 20 °C, the *Schizopelex* sp. larvae presented the

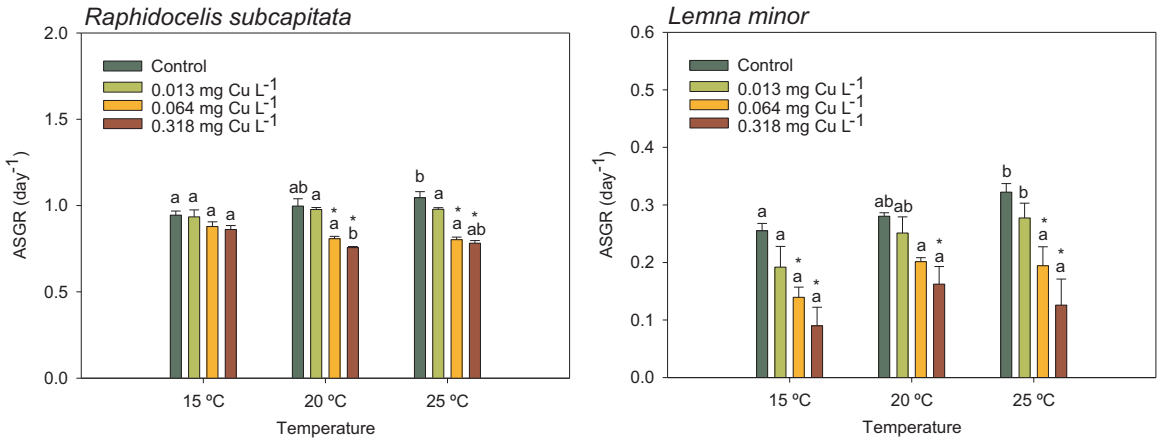

Fig. 2. The effect of copper sulfate (as mg Cu L^{-1}) contamination and increased water temperature on the average specific growth rate (ASGR) of *Raphidocelis subcapitata* and *Lemna minor*. Error bars represent standard error; asterisks (*) indicate significant differences of copper treatments relatively to the control within the same testing temperature; the lowercase letters (*a* and *b*) indicate significant differences among temperatures within each copper sulfate concentration tested.

Table 2
(A) LOEC values (mg Cu L^{-1}) and concentration of copper sulfate (as mg Cu L^{-1}) causing a 10 (EC_{10}), 20 (EC_{20}) and 50% (EC_{50}) growth inhibition of *Raphidocelis subcapitata* and *Lemna minor* for each temperature regime. The values between brackets represent the respective EC_x – 95% confidence limits. (B) Toxicity values (mg Cu L^{-1}) retrieved from the available literature. *nd* – not determined.

A

Species	Temp. (°C)	LOEC	EC10	EC20	EC50
R. subcapitata	15	-	> 0.318 -	> 0.318 -	> 0.318 -
	20	0.064	0.024 (nd – 0.069)	0.142 (0.006 – 0.277)	> 0.318 -
	25	0.064	0.009 (nd – 0.029)	0.092 (nd – 0.2)	> 0.318 -
L. minor	15	0.064	0.002 (nd – 0.008)	0.007 (nd – 0.027)	0.097 (nd – 0.233)
	20	0.318	0.007 (nd – 0.031)	0.034 (nd – 0.109)	> 0.318 -
	25	0.064	0.005 (nd – 0.020)	0.017 (nd – 0.055)	0.143 (nd – 0.309)

B

Species	Test duration	Temp. (°C)	EC50	Reference
R. subcapitata	72 h	20	0.001 (0.001 – 0.002) 0.021 (0.020 – 0.021) 0.027 (0.025 – 0.030) 0.111 (0.102 – 0.120)	Hoppe et al. 2015
	72 h	23	0.16 (0.14 – 0.18) 0.17 (0.13 – 0.22)	Paixão et al. 2008
	96 h	24	0.071 (0.045 – 0.099) 0.073 (0.049 – 0.086) 0.137 (0.090 – 0.174)	Oliveira-Filho et al. 2004
	72 h	27	0.31 (nd) 0.36 (nd) 0.55 (nd)	Rojícková et al. 1998
L. minor	96 h	22	0.47 (nd)	Khellaf and Zerdaoui 2009
	7 days	25	0.095 (0.054 – 0.150)	Naumann et al. 2007
	7 days	25	0.16 (0.11 – 0.21)	Teisseire et al. 1998

highest and the lowest GE under 0 and 0.318 mg Cu L^{-1}, respectively. Although there was an absence of significant differences among the temperatures within each copper treatment (Fig. 3, Table 3), the EC_{20} and EC_{50} values revealed a tendency for a higher toxicity at warmer conditions (i.e., 20 and 25 °C; cf. Table 4A).

4. Discussion

4.1. Primary producers growth

Previous studies reported the negative impact of copper on microalgae (e.g., Dewez et al., 2005) and macrophytes (e.g., Devi and Prasad,

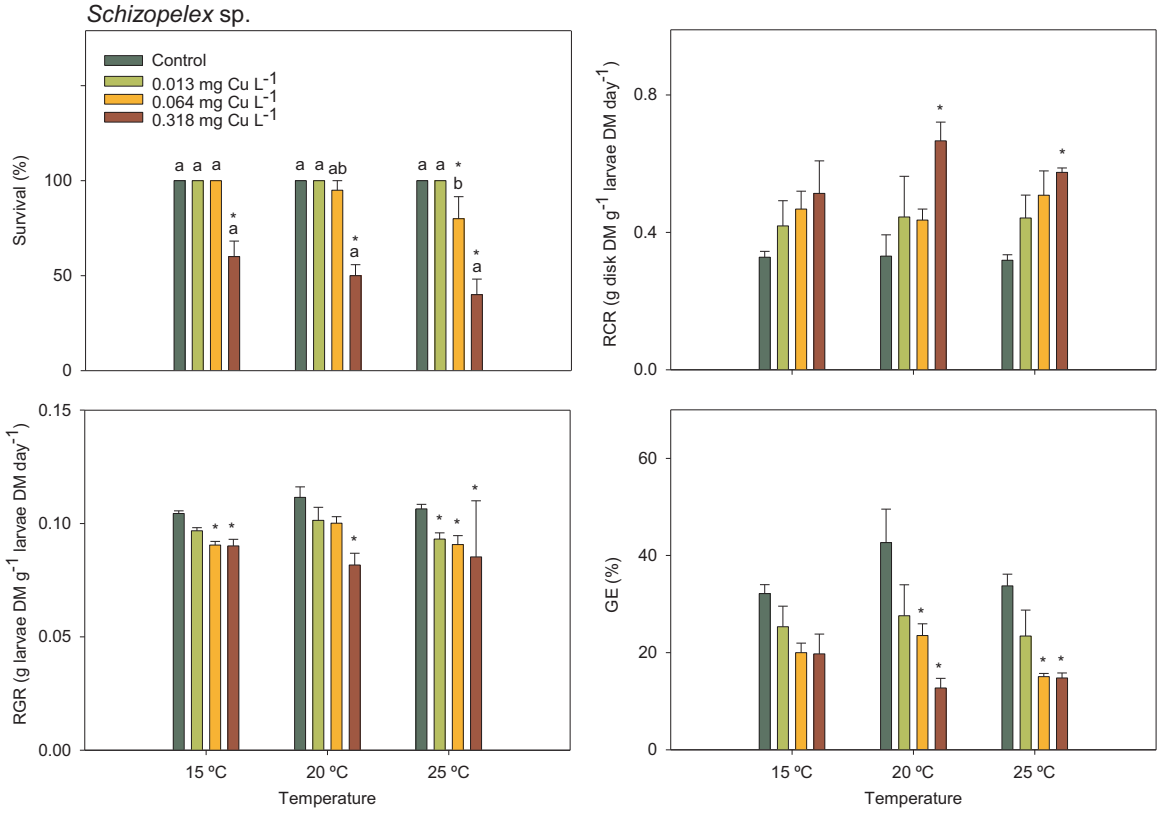

Fig. 3. The effects of copper sulfate (as mg Cu L^{-1}) contamination and increased water temperatures on *Schizopelex* sp. survival, Relative Consumption Rate (RCR), Relative Growth Rate (RGR) and Growth Efficiency (GE). Error bars represent standard error; asterisks (*) indicate significant differences between the copper concentrations and the control within the same tested temperature; and the lowercase letters (*a* and *b*) indicate significant differences between temperatures within the same copper sulfate concentration (if not signed out in the graphs, it means that there are no significant differences on shredders responses between temperatures). *DM* – dry mass.

Table 3
Summary of the two-way ANOVA applied to the survival, the relative consumption rate (RCR), the relative growth rate (RGR) and the growth efficiency (GE) data of the trichoptera *Schizopelex* sp. Significant effects are marked in bold ($p < 0.05$).

Endpoint	Source of variation	df	MS	F	*p*
Survival	Copper contamination	3	5517.1	71.241	**< 0.001**
	Temperature	2	254.842	3.291	**0.05**
	Copper x Temperature	6	96.586	1.247	0.306
	Residual	36	77.443	–	–
RCR	Copper contamination	3	0.137	8.357	**< 0.001**
	Temperature	2	0.006	0.378	0.688
	Copper x Temperature	6	0.008	0.483	0.817
	Residual	36	0.016	–	–
RGR	Copper contamination	3	8.79E−04	19.618	**< 0.001**
	Temperature	2	8.84E−05	1.974	0.154
	Copper x Temperature	6	7.25E−05	1.62	0.172
	Residual	36	4.48E−05	–	–
GE	Copper contamination	3	858.009	14.873	**< 0.001**
	Temperature	2	84.392	1.463	0.246
	Copper x Temperature	6	53.949	0.935	0.483
	Residual	36	57.687	–	–

1998; Charles et al., 2006; Zhao et al., 2015) growth associated with its interference on the transport of electrons at photosystem II, as well as on the production of reactive oxygen species (ROS) that caused oxidative stress. These impairments might have also driven the growth inhibition observed on the microalgae *R. subcapitata* and the macrophyte *L. minor* exposed to increasing copper concentrations in most temperature regimes tested. For *R. subcapitata*, a significant growth depletion occurred under 20 and 25 °C at 0.064 mg Cu L^{-1} (Fig. 2, Table 2A), which was below the maximum acceptable value for copper, as established by the Portuguese legislation for the good quality of surface waters (0.1 mg Cu L^{-1}; INAG, 2009); but slightly above the international water quality guideline for chronic copper exposures (0.051 mg L^{-1} at pH = 7.0 and 4-day exposures; US EPA, 2007). Notwithstanding, it was not possible to determine EC_{50} values for microalgae growth (> 0.318 mg Cu L^{-1}) at any of the three temperatures. In the literature, the EC_{50} values for 20–24 °C were frequently below (0.001–0.17 mg Cu L^{-1}) the maximum concentration herein tested (0.318 mg Cu L^{-1}), except in a study conducted by Rojícková et al. (1998) under 27 °C, in which they obtained similar to lower Cu toxicity (EC_{50}: 0.31–0.55 mg Cu L^{-1}). Differences in test conditions, such as the duration of exposure period, water temperature, light intensity and photoperiod can be possible factors sustaining the variability in the response of *R. subcapitata* to copper.

L. minor was highly sensitive to copper contamination, especially at 15 and 25 °C, which is in agreement with the findings of Teisseire et al. (1998) and Naumann et al. (2007) (Table 2B). Khellaf and Zerdaoui (2009) reported a lower sensitivity (0.47 mg Cu L^{-1}; Table 2B), but this could be due to the comparatively shorter exposure period. At copper levels below 0.2 mg Cu L^{-1}, however, the authors found an enhanced *L. minor* growth, a phenomenon that was not confirmed in the present study.

Increasing temperatures had significantly stimulated the growth of both microalgae and macrophytes species under non-contaminated scenarios (Fig. 2, Table 1). The growth rates of *R. subcapitata* and *L. minor* in the control at the three temperature regimes were approximate to those reported by Bouterfas et al. (2002) (15 °C: 0.72 day^{-1}, 20 °C: 0.98 day^{-1}, 25 °C: 1.36 day^{-1}) for *Selenastrum minutum*; by van der Heide et al. (2006) (26.6 °C: 0.35 day^{-1}) for *L. minor* and by Lasfar et al. (2007) (15 °C: 0.16 day^{-1}, 20 °C: ~0.32 day^{-1}, 25 °C: ~0.41 day^{-1}) for the same species.

The influence of water temperature increase on copper toxicity to primary producers was however species-dependent. For *R. subcapitata*,

Table 4
(A) LOEC values (mg Cu L^{-1}) and concentration of copper sulfate (as mg Cu L^{-1}) causing lethal (LCx) or adverse effects (ECx) on the relative growth rate (RGR) and on the growth efficiency (GE) of 10%, 20% and 50% of *Schizopelex* sp. population, upon different temperatures and 7 days of exposure. The ECx values were not determined for the relative consumption rates (RCR) since this parameter was stimulated by the agrochemical. The values between brackets represent the respective ECx-95% confidence limits. (B) Summary of toxicity values (LC_{50}; mg Cu L^{-1}) retrieved from the available literature for the survival of different shredders species (phylum Arthropoda) exposed to copper.

A

Temp. (°C)	**Survival**				**RGR**				**GE**			
	LOEC	LC_{10}/EC_{10}	LC_{20}/EC_{20}	LC_{50}/EC_{50}	LOEC	LC_{10}/EC_{10}	LC_{20}/EC_{20}	LC_{50}/EC_{50}	LOEC	LC_{10}/EC_{10}	LC_{20}/EC_{20}	LC_{50}/EC_{50}
15	0.318 (nd)	0.164 (nd)	0.184 (nd)	> 0.318 (nd)	0.064 (nd)	0.03 (nd – 0.117)	> 0.318 (nd)	> 0.318 (nd)	nd (nd)	nd (nd)	0.005 (nd – 0.039)	> 0.318 (nd)
20	0.318 (nd)	0.014 (nd – 0.1)	0.050 (nd – 0.127)	0.316 (0.256 – 0.421)	0.318 (nd)	0.047 (nd – 0.161)	0.192 (-; 0.432)	> 0.318 (nd)	0.064 (nd)	nd (nd)	0.003 (nd – 0.019)	0.063 (nd – 0.179)
25	0.064 (nd)	nd (nd)	nd (nd)	0.27 (nd)	0.013 (nd)	0.085 (0.076 – 0.094)	> 0.318 (nd)	> 0.318 (nd)	0.064 (nd)	nd (nd)	0.002 (nd – 0.009)	0.088 (nd – 0.279)

B

Class	Species	Test duration	Temp. (°C)	LC_{50}	Reference
Crustacea	*Gammarus fossarum*	96 h	14	0.188 (0.141 – 0.238)	Schmidlin et al. 2015
		96 h	18	0.135 (0.111 – 0.166)	
Crustacea	*Gammarus fossarum*	6 days	20	0.078 (0.074 – 0.082)	Zubrod et al. 2014
Crustacea	*Echinogammarus meridionalis*	96 h	20	0.036 (0.020 – 0.062)	Gama et al. 2014
Crustacea	*Atyaephyra desmarestii*	96 h	20	0.165 (0.106 – 0.251)	
Insecta	*Schizopelex festiva*	96 h	20	7.274 (4.394 – 32.498)	
Insecta	*Hydropsyche angustipennis*	48 h	20	2.51 (2.1 – 3.003)	van der Geest et al.1999
		96 h	20	0.35 (0.257 – 0.478)	
		7 days	20	0.502 (0.411 – 0.614)	

nd – not determined

the toxicity of copper was incremented under warming conditions, while for *L. minor*, it was lower at 20 °C and increased at 15 °C and 25 °C. In what concerns the microalgae, the response could be related to a stimulated metabolism driven by the raise in temperature, hence promoting chemical uptake and a consequent toxicity increase (*i.e.*, lower EC_x values). For *L. minor*, however, more efficient detoxification mechanisms together with a higher copper tolerance might have occurred at 20 °C than at 15 or 25 °C (Lau et al., 2014; Zhou et al., 2014). Hence, the lowest toxicity observed at 20 °C for macrophytes suggests that it is the optimum temperature for the physiological performance of this species under copper contamination scenarios. Under no copper contamination, as abovementioned, the optimum for *L. minor* growth was 25 °C (also verified by van der Heide et al. (2006), Lasfar et al. (2007)). However, a possible shift in its performance under lower optimum temperature might have happened upon chronic exposure to copper.

4.2. Shredders survival, growth and feeding inhibition

Copper contamination of both water and food significantly reduced the survival and growth of *Schizopelex* sp., while it surprisingly enhanced the feeding activity of living organisms, especially at higher water temperatures (Fig. 3).

The lethal toxicity of copper to *Schizopelex* sp. larvae was mostly above than that of crustacean shredders, but it was lower or similar to the reported LC_{50}'s for other Trichoptera species (see Table 4). The variation in the sensitivity of shredders species to chemicals, namely metals, has been associated with different factors. For instance, a longer exposure time may enhance metal uptake by the organisms (van der Geest et al., 1999). On the other hand, shredders belonging to different taxonomic classes may evidence varied pathways or behaviors to prevent metal stress, either related with the construction of a protective case (as in *Schizopelex* sp.), a decreased metabolic activity, or different mechanisms of metal accumulation/excretion in trichopterans (van der Geest et al., 1999; Rainbow et al., 2014; Gama et al., 2014). Consequently, a different range on species sensitivity to the acute stress of chemicals may occur.

In what concerns the combined effect of metal concentrations and temperature, Batista et al. (2012) and Gama et al. (2014) reported significantly higher toxicity of cadmium and copper, respectively, at warmer conditions, as a consequence of increased ion turnover and production of ROS. Such outcome reinforces the concerns associated with an enhanced sensitivity of organisms to metals like copper, in the context of future global warming.

The higher RCRs herein determined were in accordance with the response of the shredder amphipod *Echinogammarus meridionalis,* which preferred metal contaminated leaves to non-contaminated leaves (Quintaneiro et al., 2014). Nevertheless, the same authors observed that the decapod *Atyaephyra desmarestii* had no clear preference or avoidance for copper-contaminated leaves at levels below 0.13 mg Cu L^{-1}, though they avoided them at higher Cu levels. Hence, it suggested the existence of a trade-off between the metabolic needs of this essential trace element and the processes of internal regulation. Although *Schizopelex* sp. larvae were herein exposed to higher copper concentrations (up to 0.318 mg Cu L^{-1}), the existence of a trade-off was not observed, suggesting that the concentration of copper at which food avoidance is

observed, is species-dependent. The incremented RCR values obtained in the present study at high copper levels might have resulted from a compensatory feeding behavior, which often occurs under low quality food resources (e.g., Flores et al., 2013; Mas-Martí et al., 2015).

Despite the compensatory feeding behavior, the exposure to increasing copper concentrations resulted in significantly smaller larvae. Conradi and Depledge (1998) also observed a growth reduction of the amphipod *Corophium volutator* under copper exposure, which was the result of physiological disruptions promoted by the metal and higher allocation of energy to survival than to growth and reproduction. Such energetic investment on survival under low quality food was also observed by Flores et al. (2013) in *S. vittatum* larvae. This can partly explain the elevated consumption of contaminated disk leaves and the low growth rates of *Schizopelex* sp. larvae herein observed. Significant inhibitions of the growth of detritivore-shredder species were indeed reported for other contaminants, such as for cadmium-exposed *Limnephilus* sp. (Batista et al., 2012), lindane (organochlorine insecticide)-exposed *Gammarus pulex* (Blockwell et al., 1996), and tebuconazole (azole fungicide)-exposed *Gammarus fossarum* (Zubrod et al., 2011).

Overall, the water temperature alone or in combination with copper contamination did not result in any significant effect on the consumption of disk leaves, nor on the body size of trichoptera larvae (Table 3). This contrasted with the findings of prior studies. Batista et al. (2012), Kendrick and Benstead (2013) and Mas-Martí et al. (2015) observed that warmer conditions induced statistically higher RCR and RGR of the shredders species *Limnephilus* sp., the *Pycnopsyche gentilis* larvae, and *Echinogammarus berilloni*, respectively, which they attributed to increased metabolism and energetic demand. Garcia and Pardo (2012) and Mas-Martí et al. (2015) found that when leaf quality was low, a temperature increase had a “rescue effect” on the growth of larvae of detritivores, resulting in significantly larger organisms. Besides, although it was used *Schizopelex* sp. of similar size in the present study, this factor should be taken into account when extrapolating the impact of climate changes on shredders communities, as far as it can tailor their adaptation to temperature increments. For example, Ferreira et al. (2010) observed that small individuals of *S. vittatum* from winter cohort had lower consumption rates at 15 °C comparatively to those at 10 °C; while medium-size individuals from both winter and spring cohorts had higher consumptions at warmer conditions owing to size-dependent trade-offs between optimal temperature and energy requirements.

5. Conclusion

Increasing concentrations of copper had generally inhibited primary producers and shredder growth, as well as they had reduced shredder survival and growth. Surprisingly, the feeding activity of shredders was stimulated. Although there was a tendency for more pronounced effects under higher temperatures, significant interaction between the two evaluated factors was only detected for microalgae growth; whilst a significant influence on organisms response, irrespectively of the copper concentrations tested, was determined for the growth of macrophyte and survival of trichopterans. As such, possible impacts might arise from agricultural practices regarding the use of copper fungicides, on a future climate change scenario. The compromising of the integrity of organisms belonging to key freshwater trophic levels, partly responsible for sustaining system functioning (e.g., production of oxygen, nutrient (re)cycling, make available energy/carbon sources and food/habitat/shelter for other organisms), may end up in bottom-up effects on the aquatic ecosystems, hence posing some concerns related with their balance.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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